

**Culture of Primary Epithelial Adenoma Cells from Familial Adenomatous Polyposis Patients**

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Abstract. Background: Colorectal tumors arise from unregulated cell proliferation of the intestinal epithelium through a multistep process the first step usually being premalignant adenomas. Familial adenomatous polyposis patients carry a germ line mutation in the APC gene leading to the development of thousands of polyps, which, if left untreated, lead to cancer. The goal of this study was the establishment of conditions for the culture of epithelial cells composing an adenomatous structure. Materials and Methods: All colorectal specimens were obtained from FAP patients within 1-2 hours of surgery. Cells were cultured by standard procedures. Characterization was carried out by immunostaining with pancytokeratin, vimentin and desmoplakin antibodies. Results: A culture protocol that gave rise to epithelial cell growth with high efficiency and efficacy was established. Successful subculturing of the cell sheets took place only when dispase prepared in Ca²⁺ and Mg²⁺ free medium, was used to digest polyp tissue taken from FAP patients. By using immunostaining these cells were characterized as epithelial. Conclusion: The protocol we developed here provides a means of preparing cell cultures from human colorectal adenomas, which aid in the research of the transition from adenoma to carcinoma.

The large intestine is mostly composed of epithelial cells, which undergo cell differentiation giving rise to the different epithelial cell phenotypes, essential for the characteristic intestinal morphology (1). Colorectal tumors arise from unregulated cell proliferation of the intestinal epithelium through a multistep process (2, 3). In most cases, premalignant adenomas (called polyps), which are tissue outgrowths presenting a disordered structure, are the first step to cancer development (4).

These structures have a premalignant cell morphology, which is mostly mediated by mutation in specific genes (5). In the case of the rare syndrome, familial adenomatous polyposis (FAP), patients carry germ line mutations within the APC gene (6), causing loss of function and deregulation of the Wnt signaling pathway (7). The characteristic phenotype of these patients is the existence of multiple polyps in their large intestine. If these are left untreated, cancer will develop (8). This adenoma to carcinoma transition holds important information regarding the mechanisms leading to colorectal carcinogenesis.

The goal of the study was to establish a culture protocol that would give rise to growth of adenomatous epithelial cells taken from FAP patients, which might be used in further studies of the adenoma to carcinoma transition.

Materials and Methods

Clinical specimens. All colorectal specimens, polyp and adjacent normal tissue, were obtained from familial adenomatous polyposis patients. In most cases, samples were handled within 1-2 hours after their surgical removal.
Culture media. All colorectal specimens were directly transferred to biopsy medium, consisting of high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20 mM HEPES, 200 U/ml penicillin, 200 μg/ml streptomycin, 2.5 μg/ml fungizone and 100 μg/ml gentamycin. Digestion medium consisted of 0.2% trypsin and 50 μg/ml gentamycin prepared in Ca²⁺ and Mg²⁺-free Hank’s balanced salt solution (HBSS) medium. Washing medium consisted of phosphate buffered saline (PBS) supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 5 μg/ml fungzone and 500 μg/ml gentamycin. The primary growth medium consisted of DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 50 μg/ml gentamycin, 0.25 ng/ml EGF, 1% insulin-transferrin-selenium (100x) and 2 mM L-glutamine. All culture dishes and plates (Costar, Cambridge, MA, USA) were coated with a solution of 10 μg/ml human fibronectin (Sigma, Poole, UK) and 0.14 ng/ml bovine serum albumin (Sigma, Poole, UK) in DMEM. Coating was obtained by the addition of 1 ml of the mentioned solution per well of a six-well plate and leaving it to dry overnight at 37°C. Cells or cell colonies became attached to the coated plates 16 hours after culture. All reagents were purchased from Gibco, Paisley, UK, unless otherwise stated.

Preparation of adenomas. Tissue specimens were transferred immediately, in biopsy medium, to the laboratory after surgery where they were washed four times in washing medium. Each specimen was cut into small pieces of approximately 1 mm³. Adenoma pieces were then seeded in the coated 6-well plates at a density of 2-3 pieces per well and incubated at 37°C in 5% CO₂ in air.

Subculture procedures. From our preliminary experiments, it was quite clear that the primary adenoma-derived epithelial sheets could not expand sufficiently to totally cover the culture dish.

Subculture of these cells was not successful using the classic trypsin-EDTA procedure. Therefore, dispase (2 U/ml), prepared in Ca²⁺ and Mg²⁺-free Hank’s balanced salt solution (HBSS) medium, was used. Dispase solution was added to the sheets of cells for 10 to 20 minutes at 37°C, until the cell sheets detached from the culture dish as layers and not as single cells. They were then pelleted by centrifugation and subcultured into new plastic dishes in primary growth medium.

Immunostaining procedures. Cells were immediately transferred from culture to ice and were fixed with the addition of 3.7% formaldehyde for 8 minutes. An ice-cold mixture of 8:1 methanol:acetone (v/v) was added for 10 minutes. Cells were then blocked in 5% FBS prepared in PBS for 30 minutes at room temperature. They were primarily incubated with FITC-conjugated anti-pancytokinin (Sigma, Poole, UK), FITC-conjugated anti-vimentin (Santa Cruz Biotechnology Inc, Heidelberg, Germany) or FITC-conjugated mouse IgG DP 2.15 (desmoplakin) (Acris, Herford, Germany) antibodies at dilutions of 1:300, 1:600 and 1:100 respectively. The primary antibodies were prepared in 1.5% FBS in PBS for 16 hours at 4°C. Lastly, the secondary antibody Alexa Fluor 488 goat anti-mouse was added, in which cells were incubated for 1 hour in the dark at room temperature. Cell nuclei were further stained with 10 μg/ml Hoescht No. 33342. Cells were extensively washed with PBS between steps.

Results

The phenotype of the obtained cultures. When adenoma samples were dissected and directly cultured in the BSA-fibronectin-coated dishes, clusters of five to ten round cells were observed three days after the culture initiation. They seemed to be compact and strongly connected to each other. Changing the media removed all the unattached cells, while attached clusters slowly expanded. After one month, piling up colonies of epithelial cells was observed (Figure 1). Confluency was never observed in these cells. It was not possible to obtain any cultures when the colonic tissue specimens were digested with collagenase. This was probably due to the fact that the integrity of desmosomal bonds between the cells was disrupted by the collagenase.

In the case of the normal colonic samples, some single round cells were isolated and were apparent from the second day of culture. After the fifth day of culture, these cells started to detach from the culture dish, migrating to the supernatant, where they died. It was not been possible to maintain these cells in culture for more than 10 days.

Culture and subculture conditions. Overnight enzymatic digestion of the adenomas using collagenase Type IA (1.5 mg/ml) (Sigma, Poole, UK) did not result in epithelial cell isolation in any case. Four adenomatous specimens have been received in total, two of which have been bacterially contaminated. Two cell lines have been initiated from the other two specimens, derived from the colon of polyposis patients.

All cell cultures were initiated and maintained in primary growth medium, which was replaced with fresh medium every three days. Cells were not subcultured during the first three weeks. Additionally, it was not possible to subculture these cell sheets using either the classic method of trypsinization or scraping. This may be due to breakage of the desmosomes, which hold the cells together. Figure 2 shows the desmosomal immunostaining in these cells. Successful subculturing of the cell sheets took place only when dispase prepared in Ca²⁺ and Mg²⁺ free medium was used. This procedure seems to preserve the integrity of the desmosomal bonds between the cells. Cells were successfully passaged 4 times and maintained in culture for approximately 114 days, before they die.

Cell characterization. In order to determine the nature of the cells, they were stained with the pancytokerin antibody, which specifically stains filaments of the cytokeratin type. These filaments are characteristic in cancer cells of epithelial origin while vimentin is present in mesenchymal-sarcoma cells. Positive staining with anti-pancytokerin (Figure 3) and negative staining with anti-vimentin strongly suggested the epithelial nature of these cells. The exact
Figure 1. Nuclear staining of primary adenoma epithelial cells from patients with polyposis. Piling up colonies as shown under phase-contrast microscopy (A, x20 magnification) and Cell nuclei stained with Hoescht (B, x20 magnification).

Figure 2. Desmosomal immunostaining of the isolated adenoma cells. (A) Cells as shown under phase-contrast microscopy, x20. (B) Cell nuclei were stained with Hoescht. Staining with the FITC-conjugated desmosomal antibody, which stains specifically desmoplakin, illustrates the existence of multiple desmosomes between these cells. Merging of the Hoescht and desmosomal staining, shows (highlighted by arrow) that cells are surrounded by desmosomes.

Figure 3. Pancytokeratin immunostaining of the isolated adenoma cells. Primary adenoma cells from patients with polyposis were stained using FITC-conjugated pancytokeratin, while nuclei were stained with Hoescht. Positive staining with cytokeratin confirms the epithelial nature of these cells.
structure of these sheets of cells was further characterized by staining with desmosomal antibody, which stains specific intracellular junctions, the so-called desmosomes, which provide sites of membrane attachment between epithelial cells (Figure 2).

Discussion

Colorectal cancer is the third most frequent type of cancer in the western world (9). In most cases, premalignant adenomas constitute the first event of colorectal tumorigenesis, through a set of transformation mechanisms. The main goal of this study was the isolation and characterization of these adenomatous cells and additionally, the establishment of particular protocols in order to enhance their routine culture. This is the essential first step which will help us to further investigate the underlying mechanisms promoting the cell changes that lead to carcinogenesis.

The in vitro isolation and culture of epithelial cells is generally a difficult task, particularly for these specific cells. Previous publications (2, 10) have reported the successful culture of these cells, but only with the use of fibroblast feeder cells which secrete particular factors enhancing cell growth. Although the coexistence of fibroblasts and adenoma epithelial cells in culture seems to be quite beneficial for the outgrowth and establishment of the latter, it is probable that these secreted factors may interfere with the underlying mechanisms leading to carcinogenesis and therefore may alter the sequence of events that actually take place in vivo. In this report, primary adenoma cells from familial polyposis coli patients were successfully isolated and maintained in culture for many days without the use of feeder fibroblasts, giving us the opportunity to characterize them.

An interesting finding of this work was the piling up morphology of the colonies of these cells. Earlier reports (11) have highlighted the importance of the three-dimensional growth of these cells. This kind of growth may be maintained by the existence of tight intracellular junctions, desmosomes, between these cells which was verified here by positive staining with the corresponding antibody. Desmosomal plaque is made up of proteins called desmoplakins which can only be found in epithelial cells and the myocardium and can be easily detected by specific antibodies. They provide, along with cytokeratins, a method for defining carcinoma cells (12). The existence of desmosomes may also be important for cell-to-cell signaling.

This study provides a basic tool to initiate in-depth research of the changes taking place during the transition from adenoma to carcinoma.

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References


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